PROC. BIOL. SOC. WASH.
103(1), 1990, pp. 235–247

THE FOURTH ANNUAL RISER LECTURE: THE ROLE OF PHYSIOLOGY AND BIOCHEMISTRY IN UNDERSTANDING ANIMAL PHYLOGENY

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Abstract.—On several occasions in the history of physiology and biochemistry, claims have been made of uniquely superior approaches to understanding animal phylogeny. Perhaps the most infamous episode was the putative dichotomy between the muscle phosphagens found in protostomes and deuterostomes, which later proved to be factually incorrect. I argue that physiology and biochemistry can be powerful tools in understanding animal evolution, and I give an example in which the common quaternary structure of the oxygen carrier hemocyanin in the three groups of living arthropods is strong evidence for a common origin and the integrity of the Phylum Arthropoda. The evidence is compelling, not because the character is molecular but because molecular structure can be interpreted in the context of its physiological setting in living, breathing animals. Thus the argument can be constructed in Darwinian terms and the evidence would be extremely difficult to interpret alternatively.

G. Evelyn Hutchinson has defined the phylum as a group of organisms which is not very clearly related to any other group. This definition may seem to be only humorous but in fact it is fairly accurate, both historically and conceptually. Throughout the modern history of zoology taxonomists have devised subsuming sets until they can no longer do so, and have then stopped and called the most general set a phylum. Thus it is the height of absurdity when we turn around and ask ourselves why we do not understand the relationships of the animal phyla. Ignorance has been cleverly built into the process (to keep ourselves in business?).

Nevertheless I, like many others, find the temptation irresistable. I believe that the quest makes us think about how animals work in ways that we would not if we fail to undertake it. And understanding how animals work is what physiological and biochemical zoology is all about.

Do physiology and biochemistry have anything to offer to understanding animal phylogeny that other subdisciplines do not? In one sense the answer must be affirmative because the logic is essentially tautologous. If physiology and biochemistry had nothing unique about them they would not be recognized as coherent disciplines. The more meaningful question is do physiology and biochemistry have anything especially cogent to offer because of their unique subject matter? My answer is yes, but my reasons may surprise the reader.

Historical Episodes of Physiological and Biochemical Inquiry into Animal Phylogeny

Muscle phosphagens.—First I must illustrate the reasons why physiology and bio-

In 1985 the annual Riser Lecture was initiated by members, alumni and friends of the Marine Science Center, Northeastern University of Nahant, Massachusetts. The occasion was the official retirement of Professor Nathan W. Riser. As teacher, biologist and founder of the facility, 'Pete' Riser endowed the laboratory with a legacy—the importance of considering the whole organism regardless of one's special focus. We dedicate these annual lectures to that principle.

chemistry have not lived up to their potential in contributing to phylogenetic knowledge. The most celebrated example of an attempt to use biochemical data in the study of animal evolution involves the distribution of the phosphagens, the guanidine derivatives found in muscle and a variety of other tissues that store high energy PO_4s. In the 1920s and 30s there was something of a revolution in thinking about animal phylogeny. The early metabolic biochemists were rapidly unravelling pathways of intermediary metabolism, concentrating on somatic muscle. A "dichotomy" was perceived to distinguish "vertebrates"[1] and "invertebrates"[1]: while the vertebrates contain creatine PO_4, it was said, the invertebrates contain the structurally distant compound arginine PO_4 (Needham et al. 1932, Kutscher & Ackermann 1933, Baldwin & Needham 1937). This theory also supposed that phosphoarginine was the original, now relict form and that phosphocreatine is a relatively new molecule that arose late in animal evolution. Even more excitement was generated by the first exceptions because they were found in the deuterostomes, some of which contain both phosphagens (e.g., Baldwin & Yudkin 1950). The coexistence was regarded as an example of transition from the relict to the modern form.

A number of reviews and monographs were written using these relationships to introduce the idea that biochemistry undertaken in a comparative framework can make important contributions to phylogenetic knowledge. When Baldwin published the 1949 edition of his essay entitled *Comparative Biochemistry,* the evidence cited in support of a phylogenetic distribution of the muscle phosphagens represented six of the several dozen animal phyla. Ten years later in the final edition of his textbook, the database included about 50 species representing 9 phyla, which was considered a large sample (Baldwin 1959).

By the middle 1950s van Thoai & Robin (1969) had begun to report a number of disturbing exceptions of phosphocreatine in the wrong (viz. protostomate) animals. Nonetheless the notion that these compounds are distributed along phylogenetic lines persisted for another decade. Of some 118 species of annelids and related groups examined by van Thoai & Robin (1969), for example, more than twice as many contain the wrong phosphagen as the right one (albeit an equally large number contains compounds closely related to phosphoarginine, which were unknown to the early workers). Phosphocreatine has been found in the Porifera as well.

Thomas Kuhn (1970) wrote a book on the heuristic role of what he called paradigms in advancing scientific knowledge. Paradigms are "universally recognized scientific achievements that for a time provide model problems and solutions to a community of practitioners" (p. viii). And if the communication facilitated by such widespread concurrence is fruitful, the truth of the paradigm matters very little. In support of his thesis Kuhn (1970) noted that he would use examples from physics and chemistry because he knew them best. But he also maintained that he could have used examples from biology had he so chosen.

I have always thought that Kuhn was wrong on that point, primarily because in biology we almost never concur so widely that we communicate without a lot of argument. But the phosphagen theory was about as close to a paradigm as biology ever gets.[2]

[1] I remain amazed by our persistence in employing, not only in our biological curricula but also in our original researches, this particular pseudo-dichotomy, the fallacy of which has been recognized for a century and a half!

[2] The only other examples that come to my mind of paradigms that proved to be incorrect are the biogenetic law and perhaps, insofar as the principles are regarded as generally true, *Drosophila* genetics.

As early as 1946 Greenwald had found the wrong phosphagen in annelids and echiuroids. Instead of concluding that something might be wrong with the theory, however, he concluded that the annelids and echiuroids are more closely related to the chordates than previously supposed! When later investigators failed to confirm his original report of both phosphagens in the hemichordate *Balanoglossus,* Baldwin (1953) acknowledged that the original finding might be wrong (the field had always been plagued by inadequate analytical methods). But, he also suggested, in the intervening 20 years perhaps a mutation has occurred and the relict compound has now been replaced by the modern form. Finally, in their 1958 review Ennor & Morrison wrote:

> "This suggestion (of a phylogenetic distribution) . . . had an unfortunate and inhibitory effect on biochemical thought in this field for many years, and was made in spite of the fact that there was evidence to show that some invertebrates possess creatine" (p. 664, parenthesis mine).

They then hedged by concluding that the theory might be valid within the deuterostomes!

It is difficult for those of us who were not around at the time to appreciate the impact made by the phosphagen theory. Consider, for example, the status of inquiry into animal phylogeny in the 1920s. The deuterostome grouping was by no means universally accepted, and the notion that acorn worms might be more closely related to vertebrates than to other Vermes was also distasteful to some. The phosphagen theory was welcomed by proponents of modern phylogeny as especially cogent evidence because it was biochemical. Evelyn Hutchinson has reminded me that the introduction of biochemical data irreversibly expanded zoological thinking about phylogeny and so, in that sense, it is a good example of Kuhn's (1970) heuristic though erroneous paradigm. But, I suggest, physiologists and biochemists would be remiss if we failed to conclude that there is a better way of thinking about phylogeny.

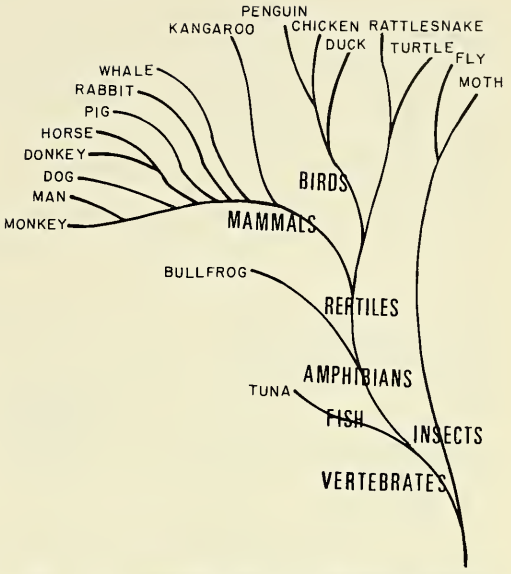

Fig. 1. Phylogenetic tree of the animal kingdom based on the primary structural similarities of cytochrome c. Redrawn from Dickerson & Geis (1969).

The theory has become a whipping boy for classical zoologists who do not want to be told that morphological data may not always take precedence. And biochemists have written a number of fairly messianic papers on how to go about the task in the right way. In my opinion, few have.

Alternative biochemical approaches.—In the 1960s the comparison of primary structure of proteins was advocated as a biochemical approach to phylogeny (e.g., Zuckerkandl & Pauling 1965). Indeed there have now been a number of such comparisons, perhaps the best known of which culminated in the phylogenetic tree based on the amino acid sequences of cytochrome c (Fig. 1). This approach, though certainly less simplistic than the phosphagen theory still has two major difficulties. One is technical and hopefully transient and the other epistemological and therefore more persistent.

Until recently, sequencing macromolecules has remained a formidable task. Cytochrome c was a good choice because it is

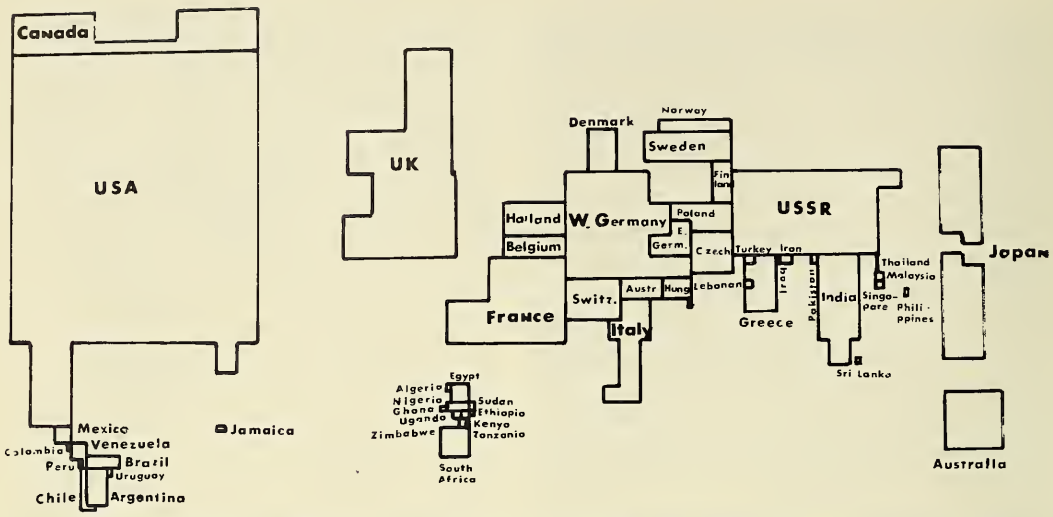

Fig. 2. A map of the world based on the frequency with which a nation's scientific journals are cited. Redrawn from Kidron & Segal (1984).

ubiquitous and it is at least not the biggest or most complex of proteins. But note the scope of the phylogenetic tree (Fig. 1). In no way does it truly depict the relationships of the animal phyla. If one were to map the nations of the world based not on their land masses but on the frequency with which their scientific journals are cited (Fig. 2), one would distort the geographic truth by a factor averaging about eight. In contrast, basing animal phylogeny on nine species of mammals, three birds, two reptiles, one amphibian, one teleost, and two insects distorts zoological truth by a factor of eighteen! Scarcity of information is no reason to reject an entire approach, but it does underscore the need to explore alternatives, unless one can suppress curiosity more than I can.

The second problem is that the nature of a putatively causal relationship between protein structure and phylogenetic relatedness is by no means clear. I will argue that the comparison of primary structures, made outside of a physiological context, tells us more about the evolution of the molecule than the evolution of the animal phyla. Once again the annelids, that perhaps most biochemically diverse of multicellular animals, can be used as an example. The primary structure of the monomeric hemoglobin in the red cells of the bloodworm *Glycera dibranchiata* has more in common with that of the hemoglobins of the distantly related vertebrates than it does with the monomeric hemoglobin in the red cells of the terebellid *Enoplobranchus sanguineus,* a member of the same class (Imamura et al. 1972, Weber et al. 1977)!

Still different molecular approaches have also been introduced. The technique of DNA pairing remains in limbo and at this writing the prospects do not appear to be good. Neither it nor immunological techniques promise to be very useful in understanding the relationships of higher taxa, for the simple reason that they require a minimum amount of relatedness that is essentially precluded by our definition of the phylum.

The very recent introduction of comparisons of sequences of nucleic acids rather than amino acids should prove to be useful. Only time and extensive validation of methodology will tell. I cannot resist the temptation to report a sense of the déjà vu, however, when I read sentences such as "the use of methods discussed here make it pos-

Table 1.—Phylogenetic relationships based on fractional structural similarity of 5S rRNA. Data from Lane et al. (1985). Mean ± SD.

	Sabellastarte japonica	*Perinereis brevicirrus*	*Solemya velum*	*Lingula anatina*
Sabellastarte japonica		0.87 ± 0.03	0.92 ± 0.03	0.88 ± 0.04
Perinereis brevicirrus	0.90 ± 0.03		0.93 ± 0.03	0.92 ± 0.03
Solemya velum	0.90 ± 0.03	0.92 ± 0.02		0.96 ± 0.02
Lingula anatina	0.86 ± 0.03	0.91 ± 0.03	0.96 ± 0.02	

sible to define the phylogenetic affiliations of any organism" (Pace et al. 1986). In fact the use of those very methods led to patently absurd conclusions, apparently unnoticed by Lane et al. (1985). The mean values for fractional "homology" (read: structural similarity) of 5S RNA indicate that the mollusc *Solemya velum* is more closely related to the annelids *Perinereis brevicirrus* and *Sabellastarte japonica* than the two annelids are to one another (Table 1)! To be more accurate by taking into account the error around the mean values, Table 1 suggests that *S. velum, P. brevicirrus, S. japonica* and *Lingula anatina* are all more or less equally related to one another.

(At this point I will confess parenthetically that I eagerly await further explication and application of the methods used in relating sequences of the 16–18S ribosomal RNA fractions, a better choice, by Field et al. (1988). The exact way in which the matrix analysis of the data was performed was unclear in the original, presumably due to space constraints specified by the journal, and consultation of the literature cited clarified only the general approach, not details that would help me understand discrepancies between the relationships depicted in the various figures. I hope that my enthusiasm for this work does not arise entirely from the agreement of the conclusions reached by Field et al. (1988) with my own phylogenetic predilections, but I am not sure.)

Why have we gone wrong?—I describe these historical incidents to illustrate what I believe are at best limited and at worst poor approaches to animal phylogeny. The databases remain small. But the comparison of muscle phosphagens and protein structures were also unsound because they lacked a Darwinian component. The practitioners never asked the question why a particular phosphagen or heme protein was selected in a particular group. The early metabolic biochemists noted that they really did not know why phosphocreatine appeared to have been selected over phosphoarginine in the deuterostomes, but there was so little discussion that they must not have regarded the point as serious. To this day the relative advantages of the various phosphagens remain a subject of speculation (Hird 1986, W. R. Ellington pers. comm.).

Centipedal Hemocyanin and Its Phylogenetic Implications

Finally, I arrive at my own subject, an example of biochemical data that are sufficiently large in number and clear in relation to physiological function that they can make a contribution in deciding a controversial question about animal phyla. The biochemical and physiological data pertain to the molecular structure and respiratory function of the hemocyanins (Hcs), the Cu-containing O_2 carriers; the phylogenetic question is the status of the Phylum Arthropoda.

Distribution and quaternary structures of the hemocyanins.—In contrast to the hemoglobins the Hcs have always been regarded as coherent in taxonomic distribution, being

found only in arthropods and molluscs. Moreover, their higher order structure differs so fundamentally that there is little doubt that arthropod and molluscan Hcs had a separate origin.

Arthropod hemocyanins: Arthropod Hcs are built as multiples of anywhere from 6 to 48 monomeric subunits of about 67–80 $\times 10^3$ d, each with a single active site (Van Holde & Miller 1982). The monomers are put together loosely. The first aggregate in assembly of the native polymer is a hexamer, which exists alone in the blood of some species. In the laboratory a hexamer can be made of only one kind of polypeptide chain, although in nature there are always two or more. If the 450 $\times 10^3$ d hexamers contain an immunologically distinctive kind of chain that tends to dimerize, two hexamers pair to form 900 $\times 10^3$ d dodecamers, the most common aggregate. The further formation of still larger aggregates remains somewhat unclear but some hypotheses suppose that a still different kind of chain is required to make the 1.5 $\times 10^6$ d 24-mers (icosatetramers, found in a number of chelicerates and a single group of crustaceans) and another still must be present to make 3 $\times 10^6$ d 48-mers (tessaracontaoctamers, found in *Limulus*).

In the arthropods especially, the progressive formation of higher order polymers is a physiologically important process because it lowers the number of osmotically active macromolecules without diminishing the O_2 carrying capacity of the blood, which allows the animal to maintain an excess of blood hydrostatic over colloid osmotic pressure and thus to form its primary urine (Snyder & Mangum 1982). Polymerization also makes more compact molecules, which minimizes viscosity and makes it easier for the animal to push its blood around.

Arthropod Hcs have a distinctive electron dense image. In high resolution micrographs hexamers look like 12.5 μm wide hexagons in top view and squares in side view. In lower resolution micrographs they appear more or less spherical. The hexagons are actually trigons of kidney-shaped monomers and there are two stacks of them in a hexamer. With only one exception the two hexamers in a dodecamer are rotated 45° with respect to one another, so they look like one hexagon and one square. 24-mers look like two pairs of spherical hexamers divided by a 2 μm cleft. The orientation of the 24-mers in a 48-mer is still somewhat uncertain. But a native 48-mer can dissociate to an aggregate that looks like a native 24-mer, which can dissociate to a native-looking dodecamer, etc. The dissociation products never include intermediate multiples, 18- or 36-mers. One would not expect to find 18-mers if the assembly process entails pairing first hexamers and then dodecamers. But there is no known structural constraint on putting a third dodecamer onto a 24-mer to make a triantahexamer.

Molluscan hemocyanins: Molluscan Hcs are built of very large (450–500 $\times 10^3$ d) monomers that have been likened to a string of beads because each contains not one but 7–8 active sites (Van Holde & Miller 1982). The 7–8 O_2 binding domains are covalently linked to one another, although the giant monomers are loosely linked together in higher order aggregates. The native polymer exists in the blood as 10 (4.5 $\times 10^6$ d), 20 (no less than 9 $\times 10^6$ d) or sometimes even more of these chains. The blood of many gastropods is opaque, not because the Hc is so highly concentrated but because it scatters so much light. We routinely prepare these molecules, incidentally, by spinning them down in a not especially powerful ultracentrifuge. In electron micrographs they look like cylinders of various heights depending on degree of polymerization, and the ends of the cylinders have 'collars' and 'caps.'

Distribution of the hemocyanins: In the arthropods Hcs have long been known to occur in the crustaceans and chelicerates and several years ago I suggested that they might also have occurred in the trilobites, because trilobites inhabited the O_2 poor aquatic habitat (Mangum 1980). In the molluscs they

have been found in four of the five classes examined (Polyplacophora, Bivalvia, Gastropoda and Cephalopoda), and my recent preliminary examination of the scaphopods was inconclusive (Mangum et al. 1987).

So a few years ago I was surprised to run across a report of Hc in a centipede, a member of the largely terrestrial uniramian arthropods. Sundara Rajulu (1969) had the interesting idea that an O_2 carrier might be needed in the relatively primitive scutigeromorph centipedes because it is the only group of Uniramia in which the tracheal system ends blindly in the blood rather than proceeding all the way to the tissues. His evidence was very suggestive but it did not include reversible O_2 binding, the critical property of an O_2 carrier. Perhaps for this reason the importance of the possibility had not been recognized by proponents of various points of view in the ongoing debate about arthropod phylogeny. At the time I was visiting Drs. Robert and Nora Terwilliger at the University of Oregon Institute of Marine Biology. We went out into the woods and picked up the first centipede we saw and performed an electrophoretic analysis of its blood. Sure enough the blood contained a Cu-binding polypeptide. But when we examined its absorption spectrum, it was totally different from that of a Hc. We had chosen a group of centipedes in which the tracheal system does terminate at the tissues, making a blood O_2 carrier redundant.

When I returned to Virginia I asked the resident expert where one must go to collect scutigeromorph centipedes and he pointed to the ceiling and I grabbed an individual of *Scutigera coleoptrata* and started working on it.[3]

Centipedal Hemocyanin

Structural and functional properties.—The blood of the common house centipede has the typical absorption spectrum of Hc, with a protein band at 280 and a Cu-O_2 maximum at 339 μm (Mangum et al. 1985). Following deoxygenation the absorption peak at the active site disappears and following re-oxygenation the peak reappears. Already this means that a Hc is present. Detailed measurements of O_2 binding revealed an exceptionally low oxygen affinity, which may prove to be related to high blood PO_2 in this terrestrial animal, and exceptionally great cooperativity that increases markedly with oxygenation state (Fig. 3). The Bohr shift is typically arthropod in magnitude and also normal, unlike that of some chelicerates. However, the direction of the Bohr shift varies within fairly closely related species so it cannot be regarded as taxonomically useful.

Centipedal Hc is fairly concentrated in the blood, which is typical of terrestrial arthropods, and the concentration almost certainly varies with hydration state in these desiccation-prone animals. In our original report I said that the blood, mercifully, fails to clot but this statement may slightly misrepresent the case. At least in small bore capillary tubes and at the termini of severed antennae, the blood becomes sticky and ceases to flow.

In electrophoretic procedures intended to estimate molecular size the centipede monomers co-migrate almost exactly with those of the blue crab *Callinectes sapidus* and slightly behind the smaller ones found in *Limulus*. Centipedal Hc has monomers of two different sizes, 72 and 80 $\times$ 10^3 d, figures very typical of the arthropod Hcs.

In our early electron micrographs of the native polymer we saw only four hexamers and we thought that the native polymer might be a 24-mer, which would also be very conventional. But in the meantime our collaborators Drs. K. E. Van Holde and K. I. Miller of Oregon State University performed sedimentation equilibrium measurements, and they arrived at a molecular weight estimate of 2.8 $\times$ 10^6 d, in between what we would expect for a 24- and a 48-mer. We had noticed on our gels that, unlike

[3] I later learned that my bathtub is a far better collecting site.

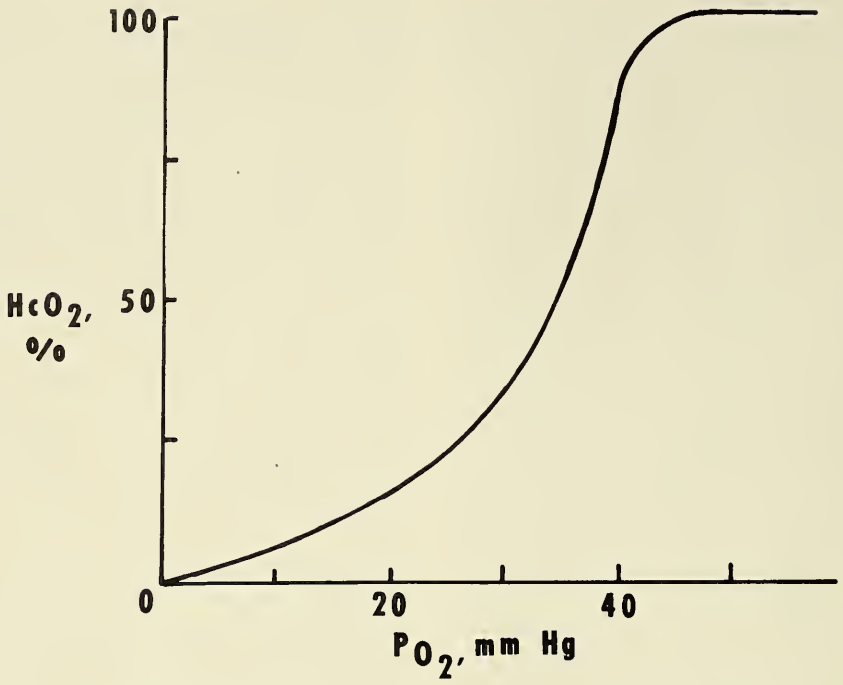

Fig. 3. O_2 binding of centipedal Hc at pH 7.5 and 25°C. 0.05 M Tris maleate buffered physiological saline. Data from Mangum et al. (1985).

C. sapidus and *Limulus,* centipede blood contains a lot of material that differs in size from typical arthropod monomers. The amount this material, 30–35% of the total, would be more than enough to bias the molecular weight estimates. We thought that centipedal Hc might consist of garden-variety arthropod monomers somehow complexed with a uniquely uniramian matrix. But when Dr. Miller purified the Hc (which was no mean feat; I feel fortunate when I collect as much as 10 μl blood from an individual, and I rarely find more than one or two animals per week and only from about May into October) and re-electrophoresed it, all of the extra material disappeared, leaving behind only the garden-variety arthropod monomers. By this time our micrographs fairly clearly showed more than four hexamers.

The centipede polymer is a triantahexamer, a multiple of 36 monomers. Initially we were quite excited about this result because, as indicated above, Hc triantahexamers had never before been found. But after contemplating it for just a little while we recognized that the aggregation of three dodecamers violates no known structural constraint. The real question is why there are not more of them.

There is one structural feature of persistent interest. The electrophoretic procedure to which I alluded above is useful in characterizing molecular weight but it is not the most sensitive. For example, *C. sapidus* Hc contains monomers of only two different sizes but it can have as many as six chains separable in the native conformation by charge (Mason et al. 1983, Mangum & Rainer 1988). But centipedal Hc still contains only two monomers when we separate the native chains by charge. So, according to some of the current models of assembly, it should not have enough different chains to

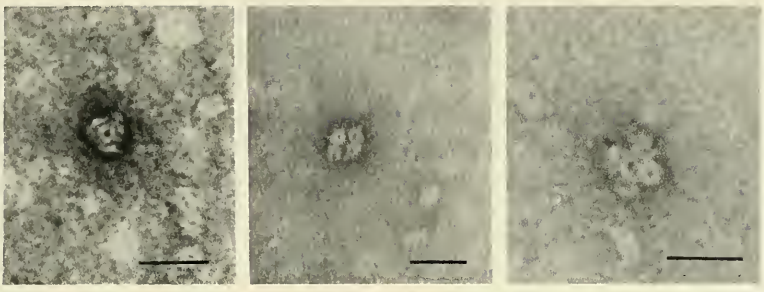

Fig. 4. Electron dense images of centipedal Hc. See text for explanation.

make the native polymer! This is of no phylogenetic interest but it does mean that the process of assembly must be rethought.

In electron micrographs we see nothing about centipedal Hc that fundamentally distinguishes it from other arthropod Hcs (Fig. 4). One image shows two pairs of spheres, each sphere measuring about 12 μm in diameter and each separated by a 2 μm cleft. Garden variety hexamers. One pair is in sharper focus than the other and one pair is slightly skewed with respect to the other. A second view shows two pairs of hexamers with a fifth in the middle. A third view shows a triangle of substructures surrounding a fourth. Two of the three peripheral units are in sharper focus than the third and, on closer inspection, additional material even more out of focus can be seen on the flat sides. More complex images, suggesting larger numbers of hexamers are also common.

All of these images can be produced by the model shown in Fig. 5. The six hexamers are arranged in an octahedral array. Two of the three pairs are lined up side by side and the third is located in an axis perpendicular to the other two. When viewed from the top or from one side only four hexamers can be seen. In this view the two pairs are not in exactly the same focus because one pair is on top and the other is underneath. When viewed from the other side five of the six can be seen. Slight tilt results in the virtual disappearance of the fifth and further tilting results in the virtual disappearance of a fourth. This is the most closed possible structure, permitting maximal interactions between the various subunits, which is consistent with the very great cooperativity of O_2 binding.

In an alternative, less closed arrangement all three pairs might be lined up side by side. But this arrangement cannot yield the image of five symmetrically placed hexamers and the image of four would show them unskewed and in the same focus. Finally, the

Fig. 5. Postulated arrangement of hexamers in centipedal triantahexamer. See text for explanation.

triangular image would not have additional material on the flat sides.

Arthropod phylogeny.—What is the phylogenetic significance of uniramian Hc? Again I must digress, to summarize briefly the various views of arthropod phylogeny.

For more than two decades Manton (e.g., 1973, 1979) argued forcefully that the so-called Phylum Arthropoda is in fact a polyphyletic assemblage of at least three and possibly four unrelated taxa. She considered the three extant groups to be of clearly different origins and noted that the extinct Trilobita may be so. Her conclusion was based on the functional morphology of the limbs, both locomotor and masticator. The sheer volume of morphological evidence she amassed in support of this inference was so formidable that few non-experts such as myself dared attempt to assess it. Her view of the living groups was supported by Anderson (1973), who compared their developmental morphology and concluded that features such as the fate map of the blastula are quite different. Because the developmental biologists among my colleagues usually decline comment on the fundamentality of blastular fate maps, I cannot assess this evidence either. The arguments were articulated so persuasively that they have even begun to appear in recent textbooks (e.g., Barnes 1987).

Recently, however, more conservative views have been revived, also persuasively. Hessler & Newman (1975) considered all of the living species of primitive crustaceans and mentally dissected away their specialized features, leaving behind only the primitive ones. From them they reconstructed a hypothetical primitive crustacean and concluded that it could have descended from a trilobite, which they also regarded as a viable candidate for the ancestral chelicerate. They argued that the Phylum Arthropoda is at most diphyletic and at least one reason for not favoring monophyly is that they did not even address the question of the origin of the Uniramia.

Most of the contributors to a volume on the subject (Gupta 1979) favored monophyly. While many of the arguments were based on a single character, taken together they are fairly cogent. Still more so are chapters by Clarke (1979), who broadly surveyed internal organ systems and came down in favor of monophyly, and Boudreaux (1979), who tried to show that 17 both derived and shared characters are not convergent. Boudreaux (1979), however, went to some lengths to emphasize that many of the 17 characters are the same ones used by Manton (1973, 1979) to support her case, which seems to me to indicate only the ease of alternative interpretation and thus to weaken his case.

The importance of Uniramian Hc in arthropod phylogeny.—By far the simplest interpretation of our findings is that the native arthropod Hc polymer is an 18th derived and shared character with a common, single origin. In each of the three living groups the native polymer is built of polypeptide chains of about the same size and linked to one another in the same way. In the uniramians, the crustaceans and all chelicerates except *Limulus*, the size of the chains is essentially identical; thus the monomers may be primitive features. In each group the hexamer, the basic functional unit that exhibits many of the critical respiratory features has the same size and the same appearance. The hexamer may also be a primitive character. But, the arrangement of hexamers in larger aggregates is distinctive and characteristic of the particular group. Recently, the homology (in the strict evolutionary sense) of the crustacean and chelicerate Hcs has been further documented by elegant and quite compelling structural evidence (Linzen et al. 1985, Markl 1986).

My inference does not clearly predict the condition of the blood in the onycophorans, which are variously regarded as more or less peripheral to the mainstream of uniramian evolution. H. D. Ellerton (Ellerton et al. 1983 and pers. comm.) could find none but he does not believe that his evidence is com-

Table 2.—The relationship of the respiratory properties of selected arthropod and molluscan Hcs. Data from Mangum & Lykkeboe (1979), Mangum (1983), Mason et al. (1983), Diefenbach & Mangum (1983), Mangum & Burnett (1986) and Mangum (unpubl.).

	Busycon canaliculatum	*Limulus polyphemus*	*Callinectes sapidus*
Bohr shift	reversed	reversed	normal
O_2 affinity at physiological pH	moderate	moderate	moderately low
Cooperativity	moderate	moderate	very great
Inorganic ion sensitivity	broad	broad	narrow
L-lactate sensitivity	none	none	great
Urate sensitivity	none	none	great
CO_2 sensitivity	indirect	indirect	direct

pletely decisive. Sundara Rajulu (pers. comm.) suspects that Hc may be there. If so, it will be most exciting to examine its quaternary structure.

My hypothesis does require the existence of Hc in the Trilobita. The elemental composition of trilobite fossils would be most interesting to learn, if it is technically possible.

The role of biochemical evidence in a physiological context.—Is this character more persuasive than others because it entails molecular structure and is therefore "closer to the gene"? Of course not—certainly not quaternary structure. But I suggest that it is more persuasive than Manton's and Boudreaux's morphological characters for two reasons. First, as I will show below, on good Darwinian principles this evidence cannot be interpreted alternatively. Second, we know a great deal about these molecules and how they work in living, breathing animals and so we know which features are and are not critical to respiratory function and hence subject to natural selection. The database on which my summary of structure is based consists of almost 200 Hcs. We have O_2 binding data obtained under fairly physiological conditions for about 150 arthropod and 80 molluscan Hcs. We have information on in vivo blood PO_2, pH and oxygenation state for probably several dozens of arthropods though, regrettably, only perhaps one dozen molluscs. We know how much oxygenation occurs at the gill and why, how much deoxygenation occurs at the tissues and why, and what environmental and physiolgical challenges perturb the system and how much and why. We know how big a role the Hc plays in total aerobic metabolism, that most fundamental process in life. We can make quantitative predictions of the hypothetical consequences of altering particular respiratory properties of the molecule.

Let us suppose for the moment that my conclusion is wrong, that the arthropod Hcs arose independently on three separate occasions and that their common quaternary structures are the products of convergence. If that were true, there must have been an overwhelming selection pressure for that particular structure, almost certainly to dictate particular respiratory properties. And yet no such selection pressure exists. It is very clear that the quaternary structure of a Hc does not constrain respiratory properties. I remind the reader once more that we have an actual example of a convergent Hc, the molluscan variety. And the O_2 binding properties of molluscan and arthropod Hcs, convergent molecules, can be more alike than those of two arthropod Hcs, homologous molecules (Table 2). So very similar respiratory properties can be associated with fundamentally dissimilar quaternary structures and one cannot argue that the arthropod structure was evolved repeatedly to preserve any particular respiratory properties because it simply does not do so. By far

the more probable interpretation is that the arthropod structure arose only once and that it is an inherited feature that (along with others) unites at least the three living groups in a natural, monophyletic taxon.

There must be other examples of molecules understood so well at both structural and physiological levels. I shall welcome the time when physiologists and biochemists once again regard animal phylogeny as a proper subject of inquiry.

Acknowledgments

This essay was written while the author was supported by the National Science Foundation (DCB 88-16172, Physiological Processes).

Literature Cited

Anderson, D. F. 1973. Embryology and phylogeny of annelids and arthropods. Oxford, Pergamon Press, 495 pp.

Baldwin, E. 1949. An introduction to comparative biochemistry. Cambridge University Press, Cambridge, 164 pp.

———. 1953. Biochemistry and evolution.—Society for Experimental Biology, Symposium 7:22–30.

———. 1959. Dynamic aspects of biochemistry. Cambridge University Press, Cambridge, 526 pp.

———, & D. M. Needham. 1937. A contribution to the comparative biochemistry of muscular and electric tissues.—Proceedings of the Royal Society, London B 122:197–205.

———, & W. N. Yudkin. 1950. The annelid phosphagen, with a note on phosphagen in Echinodermata and Protochordata.—Proceedings of the Royal Society of London B136:614–631.

Barnes, R. D. 1987. Invertebrate zoology. W. D. Saunders & Co., Philadelphia, 893 pp.

Boudreaux, H. B. 1979. Significance of intersegmental tendon system in arthropod phylogeny and a monophyletic classification of Arthropoda. Pp. 551–556 *in* A. P. Gupta, ed., Arthropod phylogeny. Van Nostrand Reinhold Co., New York.

Clarke, K. U. 1979. Visceral anatomy and arthropod phylogeny. Pp. 467–549 *in* A. P. Gupta, ed., Arthropod phylogeny. Van Nostrand Reinhold Co., New York.

Dickerson, R. E., & I. Geis. 1969. Structure and action of proteins. Harper & Row, New York, 120 pp.

Diefenbach, C. O. da C., & C. P. Mangum. 1983. The effects of inorganic ions and acclimation salinity on hemocyanin-oxygen binding in the horseshoe crab *Limulus polyphemus*.—Molecular Physiology 4:197–206.

Ellerton, H. D., N. F. Ellerton, & H. A. Robinson. 1983. Hemocyanin—a current perspective.—Proceedings of Biophysics and Molecular Biology 41:413–248.

Ennor, A. H., & J. F. Morrison. 1958. Biochemistry of the phosphagens and related guanidines.—Physiological Reviews 38:631–674.

Field, K. G. et al. 1988. Molecular phylogeny of the animal kingdom.—Science 239:748–753.

Greenwald, I. 1946. The presence of creatine in the testes of various invertebrates.—Journal of Biological Chemistry 162:239–250.

Gupta, A. G. 1979. Arthropod phylogeny. Van Nostrand Reinhold Co., New York, 762 pp.

Hessler, R. R., & W. A. Newman. 1975. A trilobitomorph origin for the Crustacea.—Fossils and Strata 4:437–459.

Hird, F. J. R. 1986. The importance of arginine in evolution.—Comparative Biochemistry and Physiology 85B:285–288.

Imamura, T., T. O. Baldwin, & A. Riggs. 1972. The amino acid sequence of the monomeric hemoglobin component from the bloodworm *Glycera dibranchiata*.—Journal of Biological Chemistry 247:2785–2797.

Kidron, M., & R. Segal. 1984. The new state of the world atlas. Simon & Schuster, New York, no pagination.

Kuhn, T. S. 1970. The structure of scientific revolutions. University of Chicago Press, Chicago, 210 pp.

Kutscher, F., & D. Ackermann. 1933. The comparative biochemistry of vertebrates and invertebrates.—Annual Review of Biochemistry 2:355–390.

Lane, D. J., D. A. Stahl, G. J. Olsen, & N. R. Pace. 1985. Analysis of hydrothermal vent-associated symbionts by ribosomal RNA sequences.—Biological Society of Washington Bulletin 6:389–400.

Linzen, B. et al. 1985. The structure of arthropod hemocyanins.—Science 229:519–524.

Mangum, C. P. 1980. Respiratory function of the hemocyanins.—American Zoologist 20:19–38.

———. 1983. The effect of hypoxia on hemocyanin-oxygen binding in the horseshoe crab *Limulus polyphemus*.—Molecular Physiology 3:217–224.

———, & L. E. Burnett. 1986. The CO_2 sensitivity of the hemocyanins and its relationship to Cl^- sensitivity.—Biological Bulletin 171:248–263.

———, & G. Lykkeboe. 1979. The influence of inorganic ions and pH on the oxygenation properties of the blood in the gastropod mollusc *Bu-*

sycon canaliculatum.—Journal of Experimental Zoology 207:417–430.

———, K. I. Miller, J. L. Scott, K. E. Van Holde, & M. P. Morse. 1987. Bivalve hemocyanin: Structural, functional and evolutionary relationships.—Biological Bulletin 173:205–221.

———, & J. S. Rainer. 1988. The relationship between subunit composition and oxygen binding of blue crab hemocyanin.—Biological Bulletin 174:77–82.

———, J. L. Scott, R. E. L. Black, K. I. Miller & K. E. Van Holde. 1985. Centipedal hemocyanin: Its structure and its implications for arthropod phylogeny.—Proceedings of the National Academy of Science USA 82:3721–3725.

Manton, S. M. 1973. Arthropod phylogeny—a modern synthesis.—Journal of Zoology 171:111–130.

———. 1979. Uniramian evolution with particular reference to the pleuron. Pp. 317–343 *in* M. Camatini, ed., Myriapod biology. Academic Press., London.

Markl, J. 1986. Evolution and function of structurally diverse subunits in the respiratory protein hemocyanin from arthropods.—Biological Bulletin 171:90–115.

Mason, R. P., C. P. Mangum, & G. Godette. 1983. The influence of inorganic ions and acclimation salinity on hemocyanin-oxygen binding in the blue crab *Callinectes sapidus*.—Biological Bulletin 164:104–123.

Needham, D. M., J. Needham, E. Baldwin, & J. Yudkin. 1932. A comparative study of the phosphagens with some remarks on the origins of vertebrates.—Proceedings of the Royal Society of London B110:260–272.

Pace, N. R., D. A. Stahl, D. J. Lane, & G. J. Olsen. 1986. The analysis of natural microbial populations by rRNA sequences.—Advances in Microbial Ecology 9:1–56.

Snyder, G. K., & C. P. Mangum. 1982. The relationship between the size and shape of an extracellular oxygen carrier and the capacity for oxygen transport. Pp. 173–188 *in* J. Bonaventura, C. Bonaventura, & S. Tesh, eds., Physiology and biology of horseshoe crabs. Allan Liss Co., New York.

Sundara Rajulu, G. 1969. Presence of haemocyanin in the blood of a centipeded *Scutigera longicornis* (Chilopoda: Myriapoda).—Current Science 38:168–169.

Van Holde, K. E., & K. I. Miller. 1982. Haemocyanins.—Quarterly Review of Biophysics 15:1–129.

van Thoai, N., & Y. Robin. 1969. Guanidine compounds and phosphagens. Pp. 163–204 *in* M. Florkin & B. T. Scheer, eds., Chemical zoology Vol. IV, Academic Press., New York.

Weber, R. E., C. Mangum, H. Steinman, C. Bonaventura, B. Sullivan, & J. Bonaventura. 1977. Hemoglobins of two terebellid polychaetes: *Enoplobranchus sanguineus* and *Amphitrite ornata*.—Comparative Biochemistry and Physiology 56A:179–187.

Zuckerkandl, E., & L. Pauling. 1965. Molecules as documents of evolutionary history.—Journal of Theoretical Biology 8:357–366.

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